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Adenovirus-Mediated Transfer of a Recombinant α1-Antitrypsin Gene to the Lung Epithelium in Vivo

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The respiratory epithelium is a potential site for somatic gene therapy for the common hereditary disorders α1-antitrypsin (α1AT) deficiency and cystic fibrosis. A replication-deficient adenoviral vector (Ad-α1AT) containing an adenovirus major late promoter and a recombinant human α1AT gene was used to infect epithelial cells of the cotton rat respiratory tract in vitro and in vivo. Freshly isolated tracheobronchial epithelial cells infected with Ad-α1AT contained human α1AT messenger RNA transcripts and synthesized and secreted human α1AT. After in vivo intratracheal administration of Ad-α1AT to these rats, human α1AT messenger RNA was observed in the respiratory epithelium, human α1AT was synthesized and secreted by lung tissue, and human α1AT was detected in the epithelial lining fluid for at least 1 week.

ONE OF THE HURDLES TO OVERcome in most forms of somatic gene therapy is the specific delivery of the therapeutic gene to the organs manifesting the disease. The lung presents special advantages because a functional gene can be delivered directly to the respiratory epithelium by means of tracheal instillation. The disadvantage of such an approach is due to the normal biology of the respiratory epithelium; only a small proportion of alveolar and airway epithelial cells go through the proliferative cycle in 1 day, and a large proportion of the cells are terminally differentiated and are, therefore, incapable of proliferation (*1*). In this regard, it may be difficult to transfer functional genes to the respiratory epithelium by means of vectors (such as retroviruses) that require proliferation of the target cells for expression of the newly transferred gene (*2*).

To circumvent the slow target-cell proliferation, we have used a recombinant adenoviral vector to transfer a recombinant human gene to the respiratory epithelium in vivo. Host cell proliferation is not required for expression of adenoviral proteins (*3, 4*), and adenoviruses are normally trophic for the respiratory epithelium (*5*). Other advantages of adenoviruses as potential vectors for human gene therapy are as follows: (i) recombination is rare; (ii) there are no known associations of human malignancies with adenoviral infections despite common human infection with adenoviruses; (iii) the adenovirus genome (which is a linear, double-stranded piece of DNA) can be manipulated to accommodate foreign genes of up to 7.0 to 7.5 kb in length; and (iv) live adenovirus has been safely used as a human vaccine (*3–8*).

The adenovirus (Ad) major late promoter (MLP) was linked to a recombinant human α1AT gene (*9*) and was incorporated into a replication-deficient recombinant (Fig. 1) (*5, 10*). The vector has a deletion of a portion of the E3 region (that permits encapsidation of the recombinant genome containing the exogenous gene) and a portion of the viral E1a coding sequence (that impairs viral replication) but contains an insert of an α1AT expression cassette (Fig. 1) (*10, 11*). After packaging into an infectious, but replication-deficient virus, Ad-α1AT is capable of directing the synthesis of human α1AT in Chinese hamstery ovary (CHO) and human cervical carcinoma (HeLa) cell lines (*10*).

We obtained tracheobronchial epithelial cells by brushing the epithelial surface of the tracheobronchial tree from the lungs of the cotton rat [*Sigmodon hispidus*, an experimental animal used to evaluate the pathogenesis of respiratory tract infections caused by human adenoviruses (*12*)]. The freshly removed cells infected in vitro with Ad-α1AT expressed human α1AT mRNA transcripts, as demonstrated by in situ hybridization with a ^{35}S-labeled antisense human α1AT RNA probe (Fig. 2). In contrast, no human α1AT mRNA transcripts were observed in uninfected, freshly isolated tracheobronchial epithelial cells. Human α1AT mRNA transcripts in the infected cells were capable of directing the synthesis and secretion of human α1AT, as shown by biosynthetic labeling and immunoprecipitation with a specific antibody to human α1AT (Fig. 2E). The newly synthesized, secreted α1AT was human α1AT, as shown by the fact that human α1AT (Fig. 2E, lane 3), but not cotton rat serum, blocked the antibody to human α1AT.

Ad-α1AT transferred the recombinant α1AT gene to the cotton rat lung in vivo (Fig. 3). Human α1AT transcripts were observed in the lungs 2 days after intratracheal instillation of Ad-α1AT, but not in lungs of animals that received only phosphate-buffered saline (PBS) or in lungs of animals that received the Ad5 E1a-deletion mutant, Ad-dl312 (*13*). Biosynthetic labeling and immunoprecipitation of extracellular protein from lung fragments removed from infected animals demonstrated that de novo synthesis and secretion of human α1AT also occurred (Fig. 3B, lanes 11

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through 15); this was not observed in uninfected animals (lane 10) or in animals infected with Ad-dl312. The de novo expression of the human α1AT protein lasted at least 1 week (lane 15), and the secreted human α1AT was functional, as shown by its ability to form a complex with its natural target, human neutrophil elastase (NE) (lanes 16 through 18).

Two lines of evidence demonstrated that the infection of the cotton rat lung with Ad-α1AT took place in vivo and was not the result of virus carried over into the in vitro biosynthetic analysis. First, immediate evaluation of lung tissue removed 2 and 3 days after in vivo infection with Ad-α1AT revealed human α1AT mRNA transcripts (Figs. 3A and 4). Second, evaluation of the respiratory epithelial lining fluid of cotton rats 3 days after infection with Ad-α1AT showed no evidence of infectious virus capable of directing the biosynthesis of human α1AT, as evidenced by exposure of the 293 cell line to epithelial lining fluid and ^{35}S-labeled methionine, followed by immunoprecipitation analysis in a manner identical to that used for the analysis of the α1AT biosynthesis by the lung fragments.

Evaluation of the cotton rat lung by in situ hybridization with antisense and sense α1AT RNA probes revealed human α1AT mRNA transcripts in lung cells of animals infected with Ad-α1AT, but not in those of uninfected animals (Fig. 4). The expression of human α1AT mRNA transcripts was patchy, as could be expected from the method of intratracheal administration of Ad-α1AT; more uniform expression should be achievable by modifications of vector delivery methods, such as by aerosol. Consistent with the observation that cotton rat respiratory epithelial cells were easily infected in vitro (Fig. 2), most of the transcripts were in epithelial cells; the available methodologies do not permit an accurate assessment of the distribution of expression among the multitude of epithelial cell types in the lung. Occasional grains were observed within interstitial cells.

Evaluation of the fluid lining the epithelial surface of the lungs with a human α1AT–specific enzyme-linked immunosorbent assay (ELISA) demonstrated the presence of human α1AT in animals infected with Ad-α1AT, but not in those infected with the deletion mutant virus Ad-dl312 or in uninfected animals (Fig. 5). Human α1AT could be detected at all the periods evaluated (days 1 to 7 after Ad-α1AT infection). No adverse effects were observed in the animals at any time after infection with either Ad-dl312 or Ad-α1AT. Because the methods available for administration of Ad-α1AT to the animals result in variable delivery and retention of the vector, it is difficult to make quantitative animal-to-animal comparisons. Thus, the time course for α1AT expression cannot be accurately determined at this time, although the de novo biosynthesis data demonstrate that the lung is still actively synthesizing human α1AT at day 7 (Fig. 3B, lane 15).

Our findings are relevant to gene therapy strategies for human diseases. The two most common lethal hereditary disorders of Caucasians, α1AT deficiency (allelic frequency

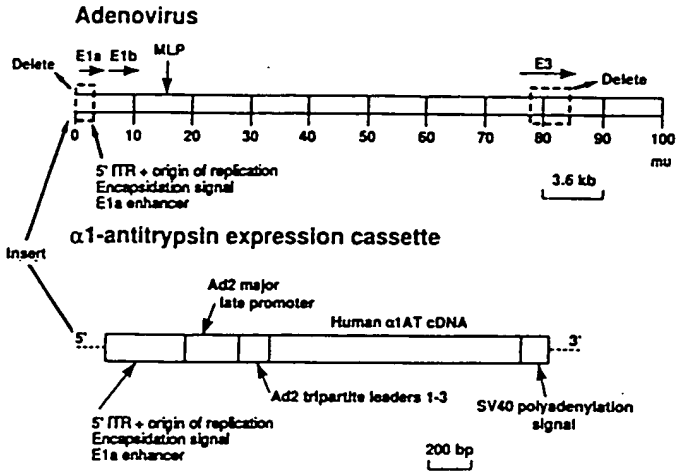

Fig. 1. Recombinant Ad vector. (**Top**) Wild-type Ad type 5 (Ad5) genome showing the E1a, E1b [map units (mu) 1.3 to 11.2; 100 mu = 36 kb], and E3 (mu 76.6 to 86.0) regions. The recombinant vector Ad-α1AT is constructed by deleting the majority of the E3 region and 2.6 mu from the left end of Ad5 and adding to the left end the α1AT expression cassette from the plasmid pMLP-α1AT, which contains regulatory sequences and a recombinant human α1AT gene (*10*). (**Bottom**) Details of the α1AT expression cassette. ITR, inverted terminal repeat. To construct the recombinant viral vector Ad-α1AT, we ligated the expression cassette with C1a I–precut Ad-dl327 DNA (*23*) (to remove a portion of the E1a region from Ad-dl327). The recombinant adenovirus DNA was transfected into the 293 cell line (*24, 25*), where it was replicated, encapsidated into an infectious virus, and isolated by plaque purification. Individual plaques were amplified by propagation in 293 cells, and the viral DNA was extracted (*26*). The intactness of the DNA of the recombinant virus was confirmed before use by restriction fragment analysis and Southern (DNA) blot. Stocks of Ad-α1AT and the Ad5 E1a deletion mutant Ad-dl312 were propagated and titered in 293 cells (*24*). The virus was released from infected cells 36 hours after infection by five cycles of freeze-thawing. For some in vivo experiments Ad-α1AT was further purified with CsCl (*25*).

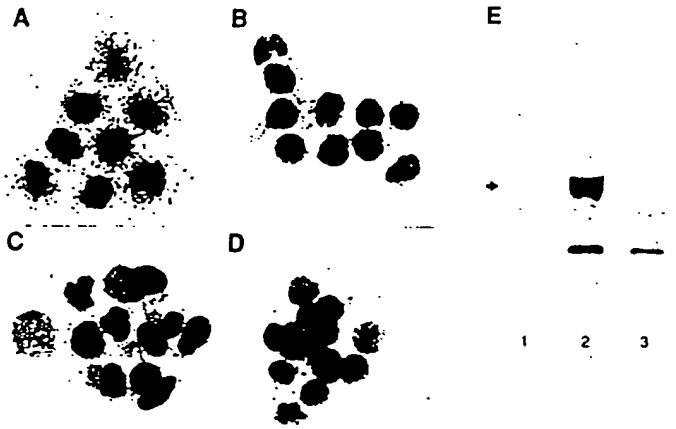

Fig. 2. Expression of human α1AT in respiratory epithelial cells freshly isolated from cotton rats infected with Ad-α1AT in vitro. (**A**) Ad-α1AT–infected cells, antisense probe. (**B**) As in (A) but for uninfected cells. (**C**) Ad-α1AT–infected cells, sense probe. (**D**) As in (C) but for uninfected cells. (**E**) Human α1AT biosynthesis and secretion. We anesthetized cotton rats (methoxyflurane inhalation), exposed the trachea and lungs through a midline thoracic incision, and perfused the pulmonary vasculature with LHC-8 medium (Biofluids) to remove blood. The trachea was transected, and the tracheobronchial epithelial cells (to the second order bronchi) were recovered with a cytologic brush. The epithelial cells were gently pelleted (300*g*, 8 min, 23°C), resuspended in LHC-8 medium, plated on fibronectin-collagen–coated plates (*27*), and infected with 2×10^7 plaque-forming units (PFU) of Ad-α1AT in LHC-8 medium or, as a control, exposed only to LHC-8 medium. After 1 day, we evaluated expression of α1AT mRNA transcripts in cytocentrifuge preparations by the technique of in situ hybridization (*28, 29*) with ^{35}S-labeled sense and antisense RNA probes (1.2×10^5 cpm/μl) prepared in pGEM-3Z (Promega). After hybridization, the cells were exposed to autoradiography film for 1 week and counterstained with hematoxylin and eosin (HE; all panels ×520). In (E), the cells were infected as in (A). After 1 day, the cells were labeled with [^{35}S]methionine (500 μCi/ml, 24 hours, 37°C), and the supernatant was evaluated by immunoprecipitation with goat antibodies to human α1AT (Cappel), SDS–polyacrylamide gel electrophoresis, and autoradiography (*30*). Lane 1, uninfected cells; lane 2, Ad-α1AT–infected cells; and lane 3, Ad-α1AT–infected cells with unlabeled human α1AT added to block the antibody. The position of migration of the 52-kD human α1AT is indicated by the arrow.

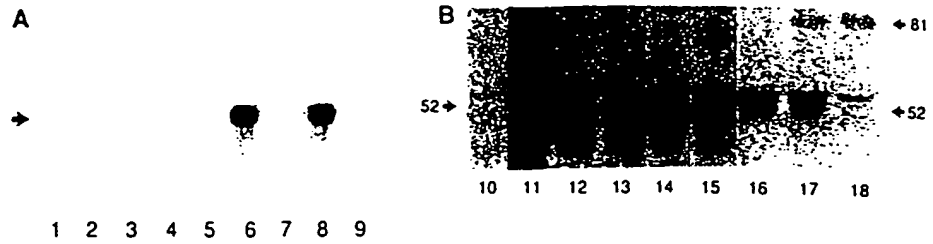

Fig. 3. Expression of human α1AT mRNA transcripts and synthesis and secretion of human α1AT by cotton rat lung after Ad-α1AT infection in vivo. Cotton rats were anesthetized, and Ad-α1AT was diluted in 300 μl of PBS to 10^8 PFU/ml and instilled into the trachea. Controls included 300 μl of PBS or 300 μl of PBS with Ad-dl312 at 10^8 PFU/ml. After 1 to 7 days, lungs were exposed and lavaged, and the pulmonary vasculature was perfused with methionine-free LHC-8 medium. (**A**) We extracted total RNA (*31*), treated the RNA with an excess of deoxyribonuclease (DNase) (RQ1 RNase-Free DNase, Promega), converted the RNA to cDNA by standard techniques, and amplified the cDNA by the polymerase chain reaction (PCR) for 25 cycles (*32*) with an adenoviral-specific primer in the tripartite leader sequences (Fig. 1) and a human α1AT exon III–specific antisense primer (*33*). PCR products were evaluated by agarose gel electrophoresis, followed by Southern hybridization with a human α1AT cDNA probe that was ^{32}P-labeled by random priming (*34*). The size of the expected fragment (1062 bp) is indicated by the arrow. Reverse transcriptase was present in lanes, 2, 4, 6, and 8. Lanes 1 and 2, uninfected lung RNA from the cotton rat (PBS control); lanes 3 and 4, 2 days after infection with Ad-dl312. Lanes 5 and 6, cotton rat 2 days after infection with Ad-α1AT; lanes 7 and 8, a different rat, treated as in 5 and 6; lane 9, PCR control without RNA or DNA template. (**B**) At various times after infection, the lungs were minced, incubated for 1 hour in methionine-free LHC-8 medium (37°C), and then incubated for 24 hours in medium with [^{35}S]-methionine (1 ml of medium/150 mg of tissue; 500 μCi/ml). The supernatant was then evaluated by immunoprecipitation with a rabbit antibody to human α1AT (Boehringer Mannheim), SDS–polyacrylamide gels, and autoradiography as in Fig. 2E. Trichloroacetic acid–precipitable radioactivity was evaluated by immunoprecipitation (for lanes 10 to 15, 2 × 10^6 cpm; for lanes 16 to 18, 1 × 10^6 cpm). Autoradiogram exposures for lanes 10 to 15 were identical; lanes 16 to 18 were evaluated at a different time; and the exposures adjusted such that the intensity of the α1AT band without NE was similar to that in lane 13. We evaluated the ability of the synthesized human α1AT to inhibit its natural substrate, NE, by incubating the supernatant with various dilutions of active NE (30 min, 23°C) before immunoprecipitation. Lane 10, uninfected control; lane 11, 1 day after infection with Ad-α1AT; lane 12, same as in 11 but with antibody exposed to unlabeled human α1AT before immunoprecipitation; lanes 13 through 15, 2, 3, and 7 days, respectively, after infection with Ad-α1AT; lanes 16 through 18, 2 days after infection with Ad-α1AT and with 3 nM, 30 nM, and 300 nM NE added to the supernatant before immunoprecipitation, respectively. The uninfected control evaluated in parallel to lanes 16 through 18 demonstrated no complexes. Indicated is the size of human α1AT (52 kD) and the human α1AT–human NE complex (81 kD).

0.01 to 0.02) and cystic fibrosis (CF; allelic frequency 0.022), have their major clinical manifestations in the lung (*9, 14*). In α1AT deficiency, mutations of coding exons of the 12.2-kb α1AT gene result in decreased serum and, hence, lung levels of α1AT, an antiprotease that normally protects the lung from destruction by the powerful proteolytic enzyme NE (*9*). Consequently, affected individuals develop emphysema by age 30 to 40, which results in a progressive respiratory impairment and a shortened life-span (*15*). Transfer of the normal α1AT gene to lung cells has the potential to protect the lung from NE by local production of the functional antiprotease.

In CF, mutations of coding exons of the 250-kb CF gene are associated with abnormalities in respiratory epithelial cell secretion of thick mucus, chronic colonization of the epithelium with pathogens such as *Pseudomonas aeruginosa,* and airway inflammation dominated by neutrophils (*14, 16*). Because the Cl^- secretory abnormalities of epithelial cells with the CF genotype can be corrected by the transfer of the normal CF gene in vitro (*17*), it should be possible to overcome the expression of the abnormal gene by transfer of the normal gene to airway epithelial cells in vivo.

A recombinant adenovirus–ornithine transcarbamylase (OTC) vector administered intravenously to *spf-ash* mice (OTC-deficient) corrected the enzyme deficiency for at least 1 year (*18*), which suggests that long-term expression is possible. In the lung, long-term expression would be aided by stable integration of the transferred gene into the appropriate stem cells (*1, 19, 20*). Production of human α1AT by lung cells continued for at least 1 week after in vivo infection with Ad-α1AT. If an inability of the virus to infect stem cells limits the length of the time of expression, repetitive administration of the recombinant virus could be used, as long as safety is ensured.

Respiratory epithelial lining fluid (ELF) levels of α1AT of 1.7 μM are sufficient to protect the human lung from its burden of NE (*21*). Because the lavage fluid used to obtain the ELF diluted the ELF 50- to 100-fold (*22*), we estimate that the actual ELF levels achieved in experimental animals with a single infection of Ad-α1AT were ~50-fold below threshold human protective level. Theoretically, it may be possible to achieve higher levels of α1AT by increasing the viral titer, delivering Ad-α1AT by aerosol (thus dispersing the vector over a broader surface area), and repeating the administrations of vector.

The safety aspects for human gene therapy of the recombinant adenoviral vectors, unlike retroviral vectors, have not been exam-

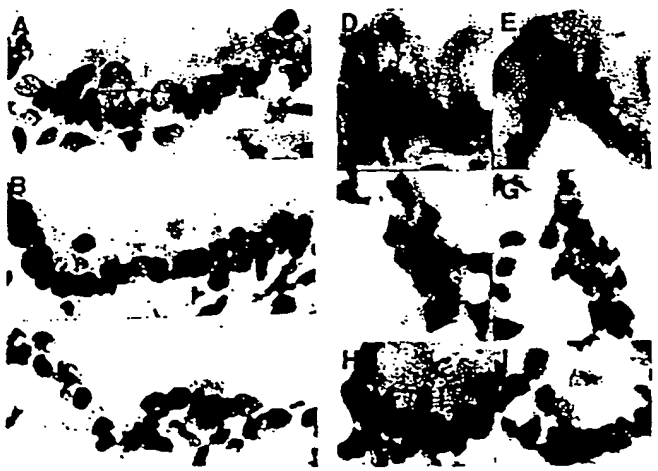

Fig. 4. In situ hybridization evaluation of lung from cotton rats infected in vivo with Ad-α1AT. (**A**) Uninfected lung (PBS control) with antisense probe. (**B** through **I**) Several examples of Ad-α1AT–infected lung. (B) Antisense probe. (C) As in (B) but with sense probe. (D) Antisense probe. (E) As in (D) but with sense probe. (F) Antisense probe. (G) As in (F) but with sense probe. (H) Antisense probe. (I) As in (H) but with sense probe. Cotton rats were infected in vivo as described in Fig. 3, with 300 μl of PBS alone or with 300 μl of Ad-α1AT diluted to between 10^8 and 10^{10} PFU/ml in PBS. After 3 days, the lungs were exposed, blood was removed by cardiac puncture, and the lungs were lavaged. The trachea and pulmonary vasculature were perfused with 4% paraformaldehyde (PFA; Fluka Chemical Corp.); the lungs were resected, fixed in 4% PFA, and frozen. Cryostat sections (7 to 10 μm) were serially treated with 0.2 M HCl and proteinase K (1 μg/ml) immediately before hybridization and evaluated with ^{35}S-labeled antisense and sense RNA probes as described in Fig. 2. As far as was possible, serial sections were used for the antisense and sense probes. All cryostat sections were stained with HE (all panels ×515).

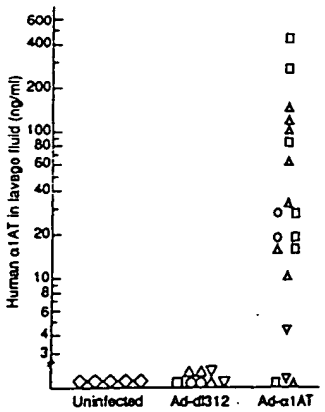

Fig. 5. The amount of human α1AT in the respiratory ELF of cotton rats after in vivo infection with Ad-α1AT. Animals were infected intratracheally with CsCl-purified Ad-α1AT (10^8 to 10^{10} PFU/ml) as described in Fig. 3; controls included uninfected animals and those infected with a similar titer of Ad-dl312. From 1 to 7 days after infection, ELF was obtained by lavage of the lungs with 2 ml of PBS. Lavage fluid was clarified (700*g*, 20 min), and the concentration of human α1AT was quantified (in quadruplicate) with a human α1AT-specific ELISA (*35*) with a sensitivity of ≥3 ng/ml. Each symbol represents the mean value of an individual animal. All uninfected animals, ◇; for infected animals, 1 (○, ○), 2 (△, △), 3 (□, □), and 7 (▽, ▽) days after infection, respectively. No α1AT was detected by ELISA in the viral preparations used for infection.

ined in detail. Safety is particularly important in weighing risk and benefit in response to α1AT deficiency, in which augmentation therapy with human plasma α1AT is available (*21*). In contrast, no definitive therapy is available for CF. Most human adults have antibodies to one of the three serogroup C adenoviruses to which Ad5 belongs (*5*). This implies little risk to those working with these vectors but may have negative implications for the virus as a gene transfer vector in the human lung. If such problems are encountered, alterations in the vector construct may be helpful. The encouraging results obtained with the Ad-α1AT recombinant adenoviral vector in vivo suggest that similar recombinant vectors may be useful for in vivo experimental animal studies with genes such as the human CF gene.

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Group I Intron Self-Splicing with Adenosine: Evidence for a Single Nucleoside-Binding Site

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For self-splicing of *Tetrahymena* ribosomal RNA precursor, guanosine binding is required for 5′ splice-site cleavage and exon ligation. Whether these two reactions use the same or different guanosine-binding sites has been debated. A double mutation in a previously identified guanosine-binding site within the intron resulted in preference for adenosine (or adenosine triphosphate) as the substrate for cleavage at the 5′ splice site. However, splicing was blocked in the exon ligation step. Blockage was reversed by a change from guanine to adenine at the 3′ splice site. These results indicate that a single determinant specifies nucleoside binding for both steps of splicing. Furthermore, it suggests that RNA could form an active site specific for adenosine triphosphate.

GROUP I INTRONS SHARE CONserved sequence elements and a common core secondary structure (*1*). Consistent with these similarities, there is a common mechanism by which group I introns are excised and the exons ligated (*2, 3*). A significant feature of all group I introns is the requirement for G (*4*) to initiate the splicing reaction (*2, 5*). The first step is a G-dependent cleavage at the 5′ splice site. This step is a transesterification (phosphoester transfer) reaction in which the substrate G is covalently joined to the 5′ end of the intron. As a result, a free 3′ hydroxyl group is generated on the U at the end of the 5′ exon. In the second step of the splicing reaction, the exons are ligated by another transesterification reaction. In this case, attack occurs at the 3′ side of the conserved G at the 3′ end of the intron (G414) (*6*) (Fig. 1). This G is essential for completion of the splicing reaction (*7*). The 5′ splice-site cleavage can be viewed as the "forward" reaction, and the exon ligation can be viewed as the "reverse" of the same reaction (*8–10*). This idea was demonstrated

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